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Detection of *Yersinia pestis* DNA in human bubo aspirates in Tanzania

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The use of molecular techniques to detect *Yersinia pestis* has enabled remarkable progress in the provision of necessary information on the occurrence of plague. In Tanzania, despite the long history of plague, DNA confirmation on the presence of *Y. pestis* in human specimens has not been done. This study was conducted in Mbulu district in Northern Tanzania where plague outbreaks have recently been reported. Nine human bubo specimens were investigated for *Y. pestis* plasminogen activator gene by using polymerase chain reaction (PCR), and two were found to be positive. The two positive amplicons, together with three previously obtained PCR positive rodent samples, were sequenced using a 3130 genetic analyzer and then compared with those available in GenBank by basic local alignment search tool (BLAST). All sequences obtained from both human and rodent samples showed 99% sequence similarity to *Y. pestis* plasmid pPCP1, detected from ancient DNA, confirming the presence of *Y. pestis* in humans that possibly sourced from rodents in Tanzania.

Key words: *Yersinia pestis*, human plague, molecular detection, Tanzania.

INTRODUCTION

Plague is a deadly infectious disease that hit the Byzantine Empire, reaching Constantinople in 542 and North Africa, Italy, Spain, and the French-German border by winter 543 (Little, 2007). The etiologic agent of plague, *Yersinia pestis*, has demonstrated a remarkable ability to spread over long distances and cause intense outbreaks interrupted by long periods of silence or reduced activity. Molecular genetic investigations have indicated that *Y. pestis* spread multiple times from foci in central Asia in greatly widening swaths as human-mediated transport became more efficient (Morelli et al., 2010; Cui et al., 2013). The disease attained its current global distribution during the third pandemic, which began in 1855 in the Chinese province of Yünnan, when it was introduced into many previously unaffected countries, via infected rats on steam ships (Vogler et al., 2013). *Y. pestis* strains have historically been classified according to their ability to utilize glycerol and reduce nitrate and have been grouped into three main subtypes or biovars as Antiqua, Medievalis, and Orientalis. These biovars can be distinguished depending on their abilities to ferment glycerol and reduce nitrate (Devignat, 1951 cited in Haensch et al., 2010).

The Medievalis biovar is unable to reduce nitrates due to

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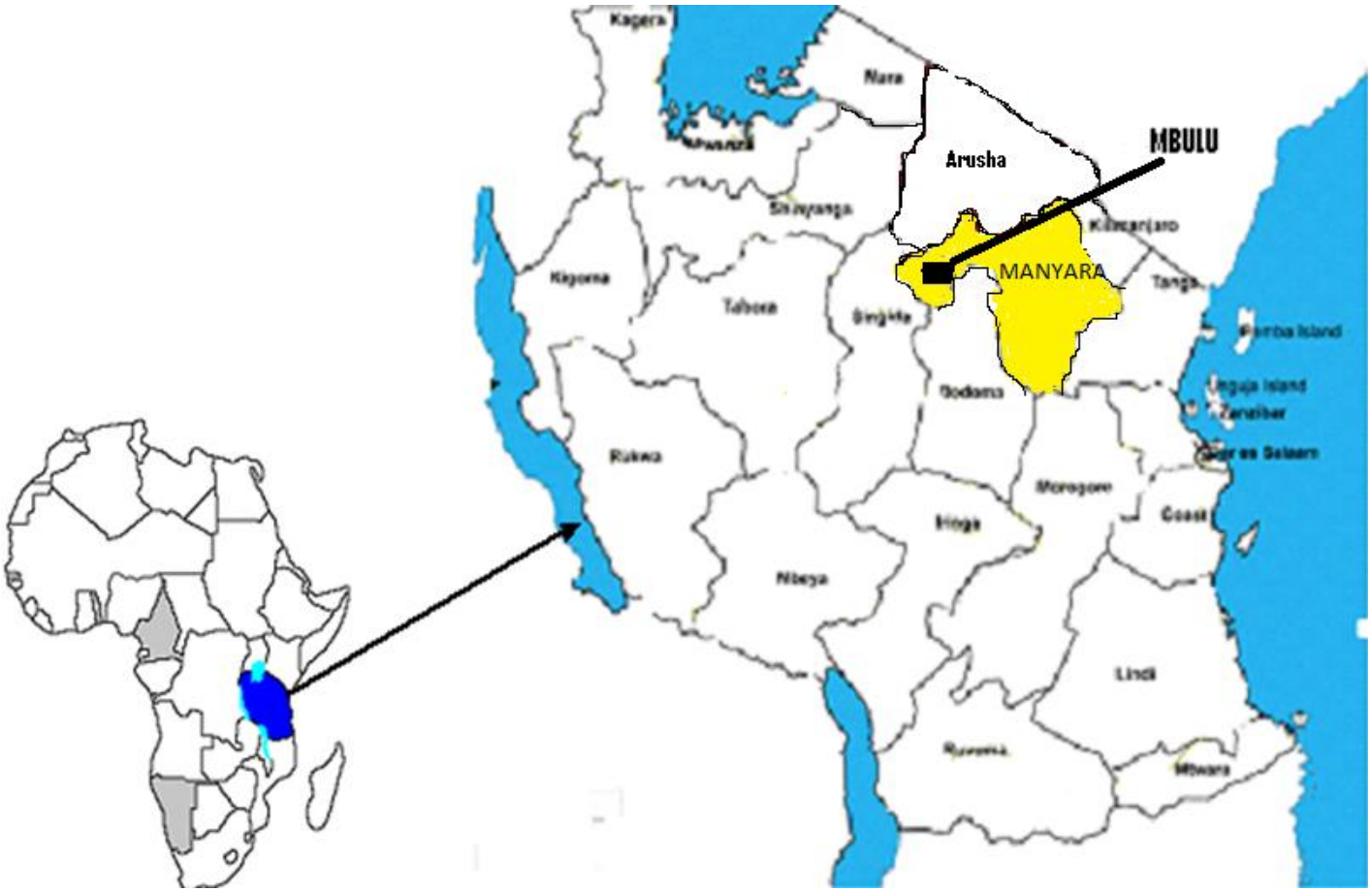

Figure 1. Map of Tanzania showing Mbulu district.

a G to T mutation that results in a stop codon in the *napA* gene (Achtman et al., 2004 cited in Haensch et al., 2010), while the Orientalis biovar cannot ferment glycerol because of a 93 bp deletion in the *glpD* gene (Motin et al., 2002; Achtman et al., 2004 cited in Haensch et al., 2010). Conversely, the Antiqua biovar is capable of performing both reactions (Haensch et al., 2010). Based on their geographic niche and on historical records that indicate the geographic origin of the pandemics, it was postulated that each biovar caused a specific pandemic (Perry and Fetherston, 1997; Wren, 2003), however, recent studies have provided direct evidence on the association of *Y. pestis* with the three historical pandemics (Bos et al., 2011; Harbeck et al., 2013). Moreover, recent collaborative efforts have provided a synthesis of old and new information describing the phenotypic and molecular diversity of *Y. pestis* (Anisimov et al., 2004; Bos et al., 2011).

In Tanzania, plague has a long history covering about 127 years, from the time it was recorded way back in 1886 believably from the Middle East (Kilonzo et al., 2005). Since then outbreaks of the disease have occurred in different parts of the country and involved large numbers of human cases and substantial case-fatality rates. During the period ranging from 1953 to 2003, 10 districts in the country reported human plague cases. Since 1980, however, only four districts (Lushoto, Singida, Karatu and Mbulu) have experienced outbreaks of the disease, and involved 8490 cases and 675 (8.0%) deaths (Kilonzo et al., 1997; 2006; Makundi et al., 2008). Despite these facts, most of the studies on plague in the country have only involved serological screening of rodents, fleas and none has utilized modern molecular tools to detect and identify *Y. pestis* directly from human patients. This study was undertaken to detect and identify *Y. pestis* from human bubo specimens in an endemic focus where cases of plague were recently reported in Tanzania (Makundi et al., 2008; allAfrica.com, 2010; Mbulu District hospital, 2011 unpublished data).

The study was conducted in Northern Tanzania in Mbulu district (Figure 1) where outbreaks of plague were reported. Mbulu is found between latitudes 3.8° and 4.5° S, and between longitudes 35° and 36° E with an altitude ranging from 1000 to 2400 m above sea level. The district

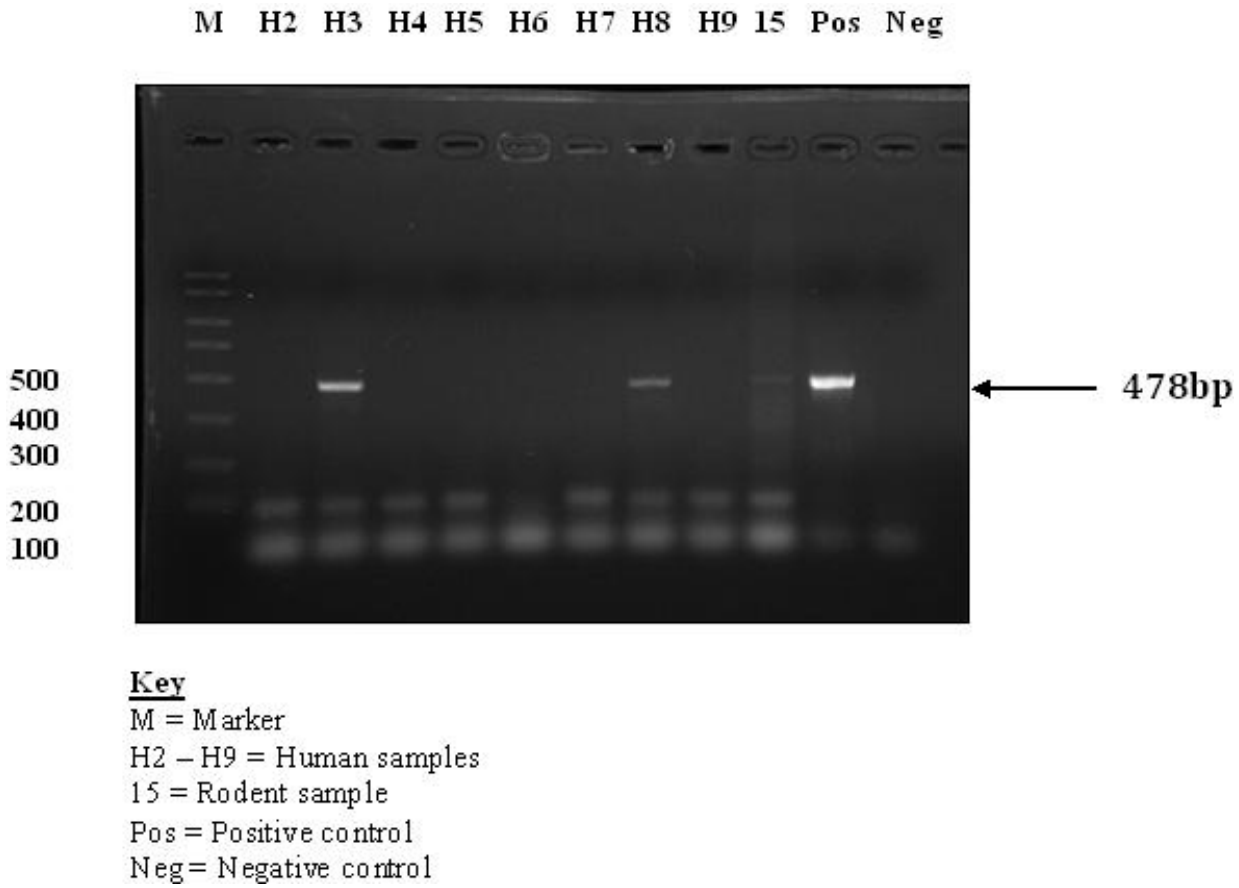

Figure 2. PCR amplification of *Y. pestis pla* gene in human bubo specimens from Mbulu district.

contains areas having semi-arid and sub-humid climate that receive annual rainfall of less than 400 mm and greater than 1200 mm, respectively (Ngowi et al., 2010).

MATERIALS AND METHODS

Bubo specimens were collected from nine patients at a local clinic during an outbreak that occurred in Mbulu district in March 2011. The bubo aspirates were inoculated in Carry Blair transport media and preserved at 4°C at Mbulu district hospital till laboratory analysis, where DNA was extracted. The volumes of the bubo aspirate were adjusted to 100 µl by adding sterile distilled water in a sterile microcentrifuge tube before DNA extraction. This was followed by the addition of the 95 µl 2X digestion buffer and 5 µl Proteinase K. The contents were mixed and incubated at 55°C for 20 min and then the total genomic DNA was extracted from the specimens using a DNA extraction kit following the manufacturer's instructions (Zymo Research, Irvine, CA, USA).

RESULTS AND DISCUSSION

To detect *Y. pestis*, specific primers previously reported (Hinnebusch and Schwan, 1993) were used to amplify a 478 bp fragment of the plasminogen activator *(pla)* gene encoded on the plasmid pPCP1. The primer sequences were *Yp* pla1: (5'- ATC TTA CTT TCC GTG AGA AG -3') and *Yp* pla2: (5'- CTT GGA TGT TGA GCT TCC TA -3') corresponding to nucleotides 971 to 990 and 1431 to 1450, respectively, of the *pla* locus sequence. The system was run in a total reaction volume of 25 µl comprising of 0.125 µl ExTaq, 2.5 µl 10XPCR buffer (Finnzyme Qy, Finland), 1.5 µl $MgCl_2$, 2.0 µl dNTP, 1 µM of forward and reverse primers each, 14.875 µl PCR water and 2.0 µl of the Template DNA. PCR amplification conditions were as follows; initial denaturation at 94°C for 1 min, subsequently 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 15 s, elongation at 72°C for 30 s and final elongation at 72°C for 5 min and then held at 4°C.

The PCR products were detected using agarose gel electrophoresis and the UV illuminator. The detected bands were subjected to sequencing. Before sequencing, the positive PCR products were purified by using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) as described by the manufacturer. The purified DNA was then sequenced directly using a Bigdye

ready reaction kit and analyzed on a 3130 Genetic Analyzer. Some *Y. pestis* positive rodent samples from our previous work (Ziwa et al., 2013) were also sequenced and compared with those available in Genbank by BLAST search. The sequences were then aligned by using the multisequence alignment CLUSTALX version 1.8.

On PCR amplification of the *Y. pestis pla* gene the expected 478 bp segment of the *Y. pestis* plasminogen activator gene was successfully amplified from 2 human bubo specimens (Figure 2). On sequencing of the two human and three *Y. pestis pla* positive amplicons from rodents, the partial sequences were found to be 99% similar to *Y. pestis* plasmid pPCP1, detected from ancient DNA (GenBank accession number HE576987.1).

This is the first study that utilized molecular tools to detect and identify the causative agent of plague in Tanzania. The ability to link a human plague isolate to a likely source has implications for investigating natural disease events (Colman et al., 2009). In our study, we linked human disease events to the likely sources of infection, a fact that has been advanced by molecular epidemiologic techniques (Colman et al., 2009). From our results, the positive amplicons from rodents and those obtained from human samples obtained during the 2011 outbreak matched, and were all identified as *Y. pestis*. This suggests the interaction of mammalian reservoirs or flea species and humans in the infectious cycle that lead to humans contacting the disease (Ebright et al., 2003; Brinkerhoff et al., 2010).

Interestingly, the partial sequences of *Y. pestis* detected from rodents during a period of no outbreak matched with the *Y. pestis* partial sequences obtained from humans during an outbreak that occurred in 2011. This further implies that the rodent strain may be responsible for the last outbreak of plague that occurred in 2011, and that the strain is being maintained by enzootic reservoir rodents during the period of no outbreak (Riehm et al., 2011). It is unclear whether the same strain may have been responsible for the other plague outbreaks that have been reported in other parts of the country (Kilonzo and Msangi, 1991; Kilonzo et al., 2005), since molecular characterization was not performed.

Conclusion

Our preliminary results have shown that the strain of *Y. pestis* which was responsible for the human plague outbreak in northern part of Tanzania has also been confirmed in rodents. In order to provide a comprehensive picture of *Y. pestis* strains in the country we recommend isolating and characterizing the bacterium in all plague foci in the country using more discriminatory molecular techniques such as multiple-locus variable number tandem repeats (VNTR) that will clearly show the dispersion routes and position of the Tanzanian *Y. pestis* in the modern phylogenetic tree of *Y. pestis*.

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